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ORIGINAL ARTICLE

Effects of commercial citrate-containing juices on urolithiasis in a *Drosophila* model

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Abstract Diet modification plays an important role in nephrolithiasis. Development of an easy, ready-to-use beverage such as a commercial juice drink to use as a preventive treatment for renal calculi formation would be widely welcomed. We previously developed a novel *Drosophila* model for the study of nephrolithiasis. It provides a new well-established drug discovery platform for this common disease. In our current study, we used the *Drosophila* model to investigate the preventive effects of various commercial juices as potential treatments for nephrolithiasis. Our results showed that apple, cranberry, orange, and pomegranate juices failed to reduce calcium oxalate (CaOx) crystal formation, whereas our positive control—potassium citrate (K-citrate)—significantly prevented CaOx crystal formation. Unlike the commercial fruit juices that were tested, the administration of K-citrate significantly ameliorated the ethylene glycol (EG)-induced life-span reduction in treated flies. These results

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indicate that EG-induced CaOx nephrolithiasis in *Drosophila* can be prevented by K-citrate, but not by commercial citrate-containing juices. However, the inhibitory capability of citrate-containing juices to reduce renal stone formation in humans requires further elucidation.

Introduction

Nephrolithiasis is a highly prevalent disease with a high rate of recurrence [1,2]. There are several factors responsible for this common disease such as genetics, diet, drugs, urinary pH, hypercalcemia, and hyperoxaluria [3,4]. Surgical treatment for kidney stones has achieved great success with minimal invasiveness; however, medication is beneficial in preventing the recurrence of nephrolithiasis [5,6]. As a major drug that alkalinizes urinary pH and increases urinary citrate levels, potassium citrate (K-citrate) may be beneficial for preventing recurrent nephrolithiasis [7,8]. However, gastrointestinal adverse effects and poor patient compliance make long-term prevention difficult. Therefore, the development of an easy, ready-to-use beverage (such as a commercial juice drink) as an alternative preventive measure is an important issue.

Diet modification plays an important role in nephrolithiasis [9,10]. Hypocitraturia is a common electrolyte disorder in urine that is present in more than 50% of patients with calcium oxalate (CaOx) stones [11]. In the urinary system, citrate is an important inhibitory substance that forms soluble complexes with calcium. Citrate reduces the saturation of calcium phosphate and CaOx, inhibits crystallization and enlargement, and thereby decreases calcium-containing stone formation [12]. Thus, providing citrate in a drink to increase urinary citrate levels may be a simple way to prevent stone formation. Citrate is abundant in citrus fruits and juices, it increases diet and beverage taste as a food modulator, and it prevents renal stone nucleation and growth [13,14]. Citrate-containing juices may be equivalent to pharmacologic K-citrate treatment because they are natural and rich sources of citrate. Research on orange juice indicates that it can prevent uric acid nephrolithiasis by alkalinizing urinary pH and decreasing urinary uric acid excretion; however, conflicting results have been reported for CaOx nephrolithiasis [15,16]. Several studies have revealed that lemonade and lemon juice are richer than orange juice in citrate levels [14,16—20], and they lower the rate of CaOx nephrolithiasis in an ethylene glycol (EG)-treated rat model [21,22]. There are many fruit juice drinks such as pomegranate, grape, fresh tomato, cranberry, blackcurrant, and apple juice that may be beneficial for preventing nephrolithiasis [23—27]. Previous researchers have mentioned their antioxidative effects [28]. Therefore, there is strong interest in the potential use of commercial citrate-containing juice drinks, distributed in the marketplace, to prevent stone formation.

A rat model in which the animals are fed the lithogenic agent, ethylene glycol, to induce the formation of CaOx crystals in the kidneys is traditionally used to mimic human kidney stone formation [29—31]. *Drosophila melanogaster* is also a good model to study human ailments because their basic biology mimics that of humans, and human diseases are easily induced in the flies through genetic manipulation [32]. We previously developed a novel *Drosophila* model for the study of nephrolithiasis. The model provides a new, well-established drug discovery platform for this common disease [33—35]. The flies are easy to breed, making this method a cost-effective alternative to the typically used rat model [36,37]. In this study, we used the *Drosophila* model to investigate the potential of various commercial juices as a preventative treatment for patients with nephrolithiasis.

Materials and methods

Drosophila urolithiasis model

This urolithiasis model was based on our previous well-established fruit fly model [33—35]. In brief, we used wild-type male fruit flies, *Drosophila melanogaster* Canton-S, as the animal model for our experiments. The flies were fed in 50-mL plastic vials containing the standard medium (the ingredients of which included corn, sugar, syrup, yeast, and agar). They were maintained under stable conditions at a temperature of 25°C, 50—60% humidity, and a 12-hour light-dark cycle. We used 0.75% (w/v) EG as the lithogenic agent, which was added to the fly medium. Various commercial citrate-containing juices (e.g., apple, cranberry, orange, and pomegranate) were added to the fly medium (25% v/v). Granules of K-citrate, which were kindly provided by Gentle Pharma (Yunlin, Taiwan), were used as the positive control. After feeding for 3 weeks, the flies were euthanized under CO_2 narcotization. The Malpighian tubules ($n \cong 150$ for each group) were dissected, removed, and processed for further examination.

Measurement of the citrate content

The electrolyte concentration in each drink was measured directly with the Roche OMNI C system (Roche Diagnostics, Indianapolis, IN). A kit employing an ultraviolet method was used to measure the concentration of citric acid with a spectrophotometer at an absorbance wavelength of 340 nm in accordance with the manufacturer's instructions (Cat. No. 0139076035; Roche, Darmstadt, Germany).

Polarized light microscopy

After dissection, the fruit flies' Malpighian tubules were observed under normal and under polarized white light with an Olympus BX51 optical microscope (Olympus, Tokyo, Japan). The crystallization of CaOx in the Malpighian tubules was graded as 0, 1, 2, or 3 ("−", "+", "++", and

"+++", respectively) through direct observation of digital photography.

Fly collection and lifespan assay

The surviving flies in each vial were counted daily and dead flies were removed. Lifespan curves were drawn according to the pooled counts of a large number of vials ($n \cong 150$). The survival rate was calculated and compared for significance by log-rank tests.

Statistical analyses

We used one-way analysis of variance to detect overall differences between the groups. For all multiple comparisons, Bonferroni correction was applied. Significantly different groups were compared pairwise by using the Mann-Whitney *U* test for crystal scores. All statistics were performed by using SigmaStat software (SPSS; SYSTAT Software, San Jose, CA, USA).

Results

Measurement of the citrate levels

Table 1 shows the citrate levels of the commercial fruit juices. Pomegranate juice contained the highest concentration of citrate (16.51 ± 0.78 g/L) and apple juice contained the least concentration (0.29 ± 0.03 g/L).

The effect of commercial fruit juices on ethylene glycol-induced calcium oxalate crystallization

Fig. 1 shows a view of the typical morphology of EG-induced CaOx crystallization in the *Drosophila* Malpighian tubules. The crystals were confirmed by scanning electron microscopy. Energy-dispersive X-ray spectroscopy was used to assess crystal refraction and to assess the relative elemental composition of the crystals. We found that EG caused crystal deposition inside the Malpighian tubules through CaOx accumulation. Fig. 2 shows the incidence of crystal formation with different commercial juices. After feeding the flies various juices in an attempt to prevent nephrolithiasis, the results showed that apple, cranberry, orange, and pomegranate juice failed to reduce CaOx crystal formation, whereas K-citrate significantly prevented CaOx crystal formation ($p < 0.05$).

Table 1 Measurement of citric acid in commercial fruit drinks.

	Citrate concentration (g/L), mean ± SD
Apple juice	0.29 ± 0.03
Cranberry juice	2.33 ± 0.02
Orange juice	2.70 ± 0.01
Pomegranate juice	16.51 ± 0.78

SD = standard deviation.

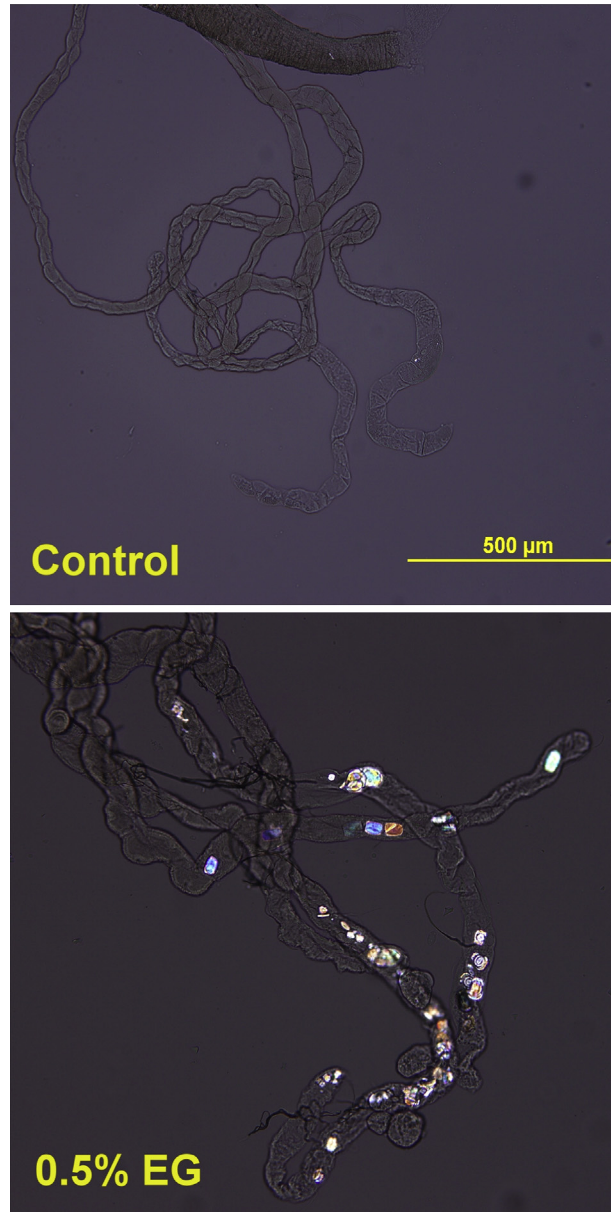

Figure 1. Ethylene glycol (EG)-induced calcium oxalate crystal deposition in the Malpighian tubules. The images show representative polarized microscopy for (top) the control flies and (bottom) the flies with 0.5% EG-induced crystal formation in Malpighian tubules. The scale bar indicates 500 μm.

Effect of commercial fruit juices on *Drosophila* lifespan

Ethylene glycol causes a significant reduction in the lifespan of *Drosophila* flies. The administration of K-citrate significantly ameliorated the EG-induced reduction in the lifespan of the treated flies, whereas the administration of commercial fruit juices failed to do the same (Fig. 3).

Discussion

Ethylene glycol-induced CaOx crystal formation in the rat is the most common nephrolithiasis model [38]. However,

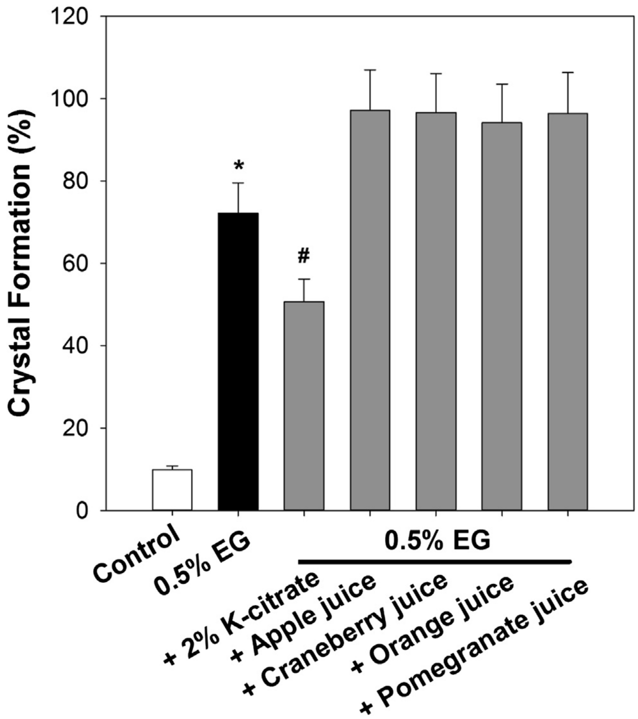

Figure 2. Crystal formation is inhibited by potassium (K)-citrate, but not commercial citrate-containing juices ($n \cong 150$ for each group). EG = ethylene glycol. *$p < 0.05$, compared to the control. $^{\#}p < 0.05$, compared to the 0.5% EG-treated group.

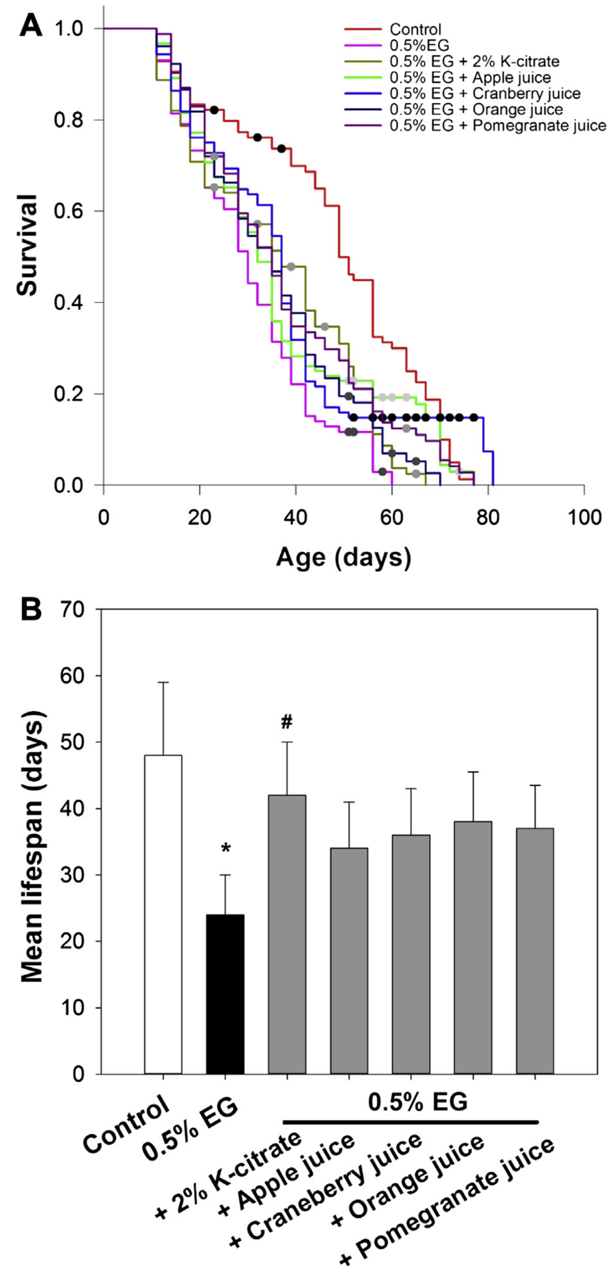

Figure 3. The effect of K-citrate and commercial citrate-containing juices on the *Drosophila* lifespan. (A) Cumulative survival distributions by the administration of EG, K-citrate, and various commercial citrate-containing juices. (B) The effect of K-citrate and various commercial citrate-containing juices on EG-induced lifespan reduction of *Drosophila* flies ($n \cong 150$ for each group). EG = ethylene glycol; K-citrate = potassium citrate. *$p < 0.05$, compared to control. $^{\#}p < 0.05$, compared to 0.5% EG-treated group.

recent studies have proved that the Malpighian tubules of *Drosophila* is an ideal new renal stone model [33,34,39]. The *Drosophila* model for nephrolithiasis progresses rapidly and the physical characteristics of the calculi are identical. Several investigators report that natural and experimental fly nephrolithiasis can mimic the human excretory system [33,35]. In the present study, testing different commercial juices in the *Drosophila* model indicated that only the positive control, K-citrate, appeared to decrease renal stone crystallization.

Several commercial juices reportedly could prevent nephrolithiasis, but in our experiment K-citrate alone decreased renal stone crystallization in the *Drosophila* model. Some investigators have shown that alkalizing fluids and high citric acid excretion could decrease the risk of CaOx stone formation [40]. Yilmaz et al. showed that fresh tomato juice may increase the level of urinary citrate, as is the case for orange juice and lemon juice. Fresh tomato juice with low oxalate and sodium levels and high magnesium levels may be useful in regard to the factors involved in the physiopathology of renal stone formation [26]. In this study, several possible reasons exist for the failure of commercial citrate-containing juices to inhibit CaOx formation, compared to K-citrate. The citrate level is relatively low in the commercial juices and K-citrate therapy requires the ingestion of many tablets daily to provide a sufficient therapeutic dose. A statistically significant increase in urinary oxalate levels in patients after orange juice intake has been demonstrated; however, this result could be attributed to the ascorbic acid, a precursor of oxalate, that may be in commercial juices [12,15]. Dietary recommendations for patients with urolithiasis include sodium restriction. The sodium concentration may also be relatively high in commercial juices.

Our study has limitations and advantages. We have utilized a new model that easily provides a large number of animals over the traditional rat model. The experimental period was short and the crystals were easily observed and calculated. However, flies are an invertebrate animal and their results may not be fully comparable with those of mammals. Translating results to the human condition by using the proposed model is somewhat difficult. For example, the absorption, metabolism, and excretion of a given substance in an insect model can be totally different from those of mammals. Therefore, investigators attempting to evaluate laboratory data to prevent nephrolithiasis must be specific in their intentions. The metabolite and fluid composition of a given substance in the Malpighian tubules of an insect model can vary greatly, compared to the metabolite and fluid composition of the substance in mammals. Therefore, the results may not be comparable. Since *Drosophila* is not an appropriate species for investigating renal function, additional evaluation methods must be established [35]. We did find that K-citrate may reduce the overall risk of renal stone formation. This outcome appears to complement the findings of different experimental models, as well as humans.

We used the *Drosophila* model in the present study as a platform for evaluating nephrolithiasis. Our results indicate that *Drosophila* flies that received K-citrate, but not commercial citrate-containing juices, experienced a reduction in renal stone formation associated with EG-induced CaOx nephrolithiasis. However, the inhibitory capability of citrate-containing juices on renal stone formation in humans requires further elucidation.

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